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***THE EFFECT OF DILUTION ON THE
STRUCTURE OF MICROBIAL COMMUNITIES***

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ABSTRACT

To determine how dilution of microbial communities affects the diversity of the diluted assemblage a series of numerical simulations were conducted that determined the theoretical change in diversity, richness, and evenness of the community with serial dilution. The results of the simulation suggested that the effects are non linear with a high degree of dependence on the initial evenness of the community being diluted. A series of incubation experiments using a range of dilutions of raw sewage as an inoculum into sterile sewage was used for comparison to the simulations. The diluted communities were maintained in batch fed reactors (3 day retention time) for 9 days. The communities were harvested and examined by conventional plating and by molecular analysis of the whole-community DNA using AFLP and T-RFLP. Additionally, CLPP analysis was also applied. The effects on richness predicted by the numerical simulations were confirmed by the analyses used. The diluted communities fell into three groups, a low dilution, intermediate dilution, and high dilution group, which corresponded well with the groupings obtained for community richness in simulation. The grouping demonstrated the non-linear nature of dilution of whole communities. Furthermore, the results implied that the undiluted community consisted of a few dominant types accompanied by a number of rare (low abundance) types as is typical in unevenly distributed communities.

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Introduction

Although microbes are capable of growing anywhere even a small amount of liquid water exists, habitats that contain organic carbon that can be used as an energy source by the microorganisms will support abundant microbial populations. The inclusion of bioregenerative life support (BLS) elements (i.e., plant growth systems and bioreactors for waste processing) will significantly increase the total abundance of microbes in extraterrestrial facilities. For example, bacterial numbers on the roots of plants within prototype hydroponic systems can be as high as 10^{11} cells g^{-1} dry wt(3). The potential exists for the communities associated with these systems (e.g., biofilms attached to plant roots or hardware surfaces, or mixed populations in the suspended phase of reactors) to harbor microbes pathogenic to humans or to the plants (14). Management of microbial communities to minimize the potential for risk to the crew and to the plants to be used for supporting the crew is an essential component of successful BLS systems.

Recommended approaches to the management of microbial communities in ALS range from strict decontamination and control to "seeding" the system with a diverse group of microorganisms (Fig. 1). One extreme would be the use of axenic plants in subsystems "bio-isolated" from the human habitat module in order to prevent the proliferation of human-associated organisms (including opportunistic human and plant pathogens) in plant growth systems. Alternatively, inoculation of plant systems with soil from fertile, disease-suppressive soils has been proposed to provide a stable microbial population that reproduces the balance in population dynamics found on Earth (1). An integrated approach involving quarantine,

sanitation, compartmentalization, and construction of microbial communities has also been recommended (13). Another approach to community management would be to use gnotobiotic communities, defined assemblages in which all of the members are known. Generation of defined mixed cultures to carry out all of the essential processes necessary for operation of BLS systems is a daunting task, and maintenance of such defined cultures without contamination from the crew or from other systems is likely impossible. Regardless of the starting mixture, evolution of the community will eventually produce something quite different from the initial assemblage. The alternative to the gnotobiotic approach invokes the ecological paradigm that diverse communities tend to be more stable and more resistant to invasion than counterparts with

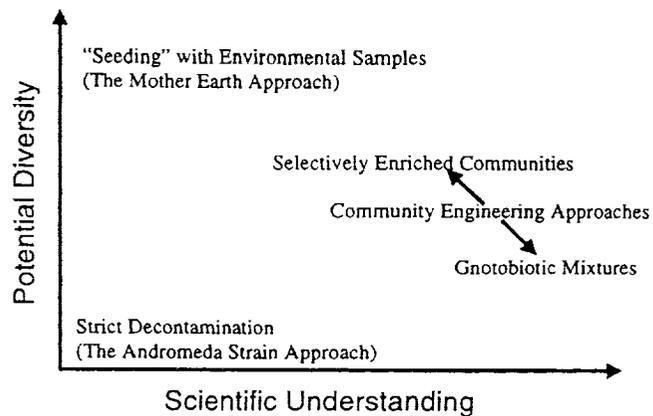


Fig. 1. Approaches to inoculum preparation for hydroponic plant growth systems and bioreactors for long term space habitation. The community engineering approaches should provide substantially greater protection of the communities against invasion by human associated bacteria or plant pathogen as compared with the strict decontamination approach (which cannot be reliably maintained for extended periods), while reducing the threat of inclusion of potentially harmful microbes as part of the inoculum. Figure reproduced from Garland, et al. (6).

few types of organisms present (e.g., 2, 7, 9, 11, 12, 15). Selection of highly diverse communities should result in narrower niches which will be difficult for pathogens or other unwanted microbes to fill. Although this concept is generally accepted by ecologists (the current emphasis on biodiversity in terrestrial ecosystems is a direct result of that acceptance), it has never been explicitly examined for microbial (i.e., bacterial) communities.

Tests of the effects of diversity on any community property or system function require a knowledge of the diversity of the community under examination, but there is no method currently available that allows diversity of a microbial community to be measured. Despite the inability to measure diversity directly, Garland (6) and Morales et al. (10) successfully used dilution to manipulate diversity for several applications. There is an inherent assumption that dilution is a linear process, even though dilution of environmental samples for microbiological analysis often produces non-linear results. The present work sought to define the relationship between dilution and resultant diversity in a series of numerical simulations accompanied by appropriate incubation experiments.

Dilution Simulations

To examine the effect of dilution on community composition, a series of numerical simulations were done. Communities were constructed by assigning each of 1×10^6 individuals a random species identification based on a normal distribution of integers from 1 to 1000 with a mean of 500 and a preset variance. By adjusting the variance, communities of different evenness could easily be simulated; low variance resulted in communities with a large number of rare species, whereas high variance yielded a community of more even distribution (Fig. 2). For the simulations described here, the total abundance and richness were held constant, and only the evenness was allowed to vary. Evenness was altered by setting the variance level to 100, 250, 1,000, and 20,000. An even distribution was simulated by creating 1000 types, each containing 1000 individuals. The initial communities were then diluted mathematically by selecting 1/10 of

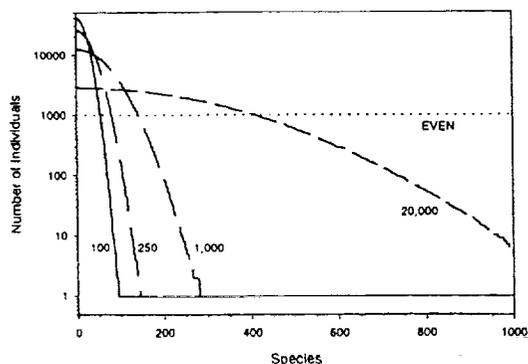


Fig. 2. Distribution of individuals among 1000 types or species as produced. These distributions are those for the initial communities used in the simulations reported here. Note that both abundance and richness are constant in the initial communities

the individuals from the array that represented the undiluted community. The species identification was placed in another array which then served as the initial community for the next dilution in the series. All serial dilutions were done in powers of 10, as is typically done for microbiological analysis. For each community and at each dilution level, the richness, evenness and diversity were calculated. Richness was taken to be the number of types in the community, diversity was expressed as the Shannon-Wiener index

$$H' = \sum_{i=1}^i p_i \ln p_i \quad (1)$$

where p_i is the fraction of the individuals in the i^{th} species.

Evenness is calculated as

$$E = \frac{H'}{H'_{\max}} \quad (2)$$

$$H'_{\max} = \ln S \quad (2a)$$

where S represents the total number of species present.

A sample dilution series for the most dominant community (var = 100) and the most even community (var = 20,000) show the type of changes in the community generated by the simulated dilutions due to difference in initial evenness (Fig. 3).

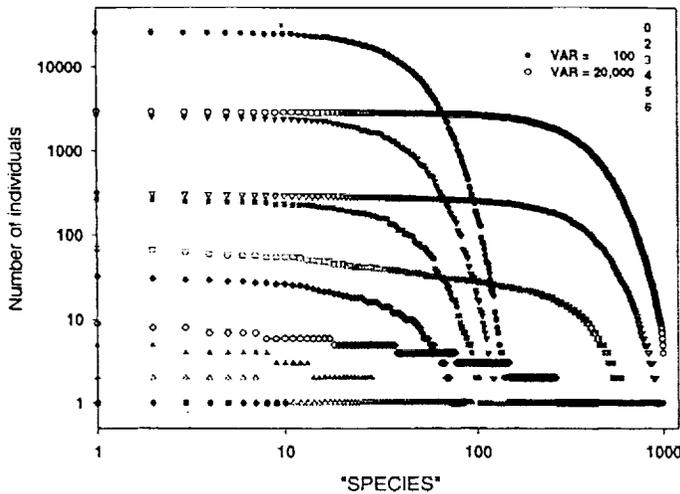


Fig. 3. Effect of dilution on species distribution in the most even (var = 20,000) and most dominant (var = 100) communities.

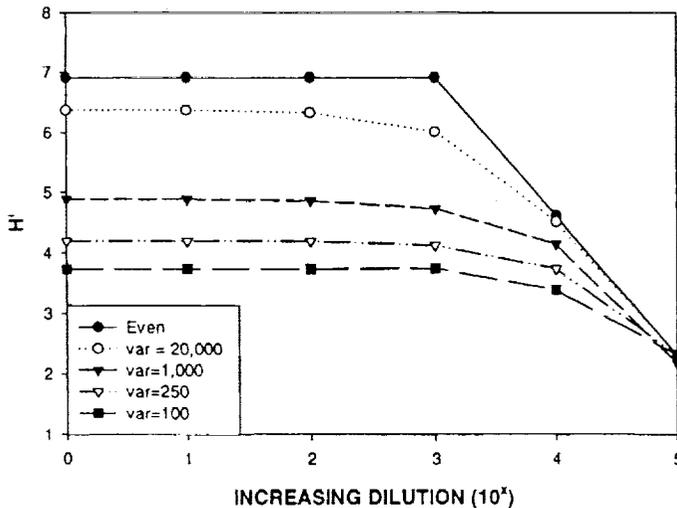


Fig. 4. Value of the Shannon-Wiener index for initial communities (0) of differing evenness and serial dilutions made from each of the communities.

created by setting var = 1000. In that case, the value of H' at the 10^{-5} dilution was 2.1640. The lower value was obtained because only 9 species were recovered in that particular simulation whereas 10 were recovered in all the others.

The serial dilution made corresponded to typical 10-fold dilutions used in microbiology, and extended from 0 to 10^{-5} . Given an initial cell concentration of $1 \times 10^6 \text{ mL}^{-1}$, the dilutions were made by selection of 1×10^5 cells from the initial community, then 1×10^4 cells from the first dilution, 1×10^3 cells from the second dilution, 1×10^2 cells from the third dilution and 1×10^1 cells from the fourth dilution. Ten cells represents the smallest number of cells in a 10-fold dilution series for which H' can be meaningfully determined (H' for 1 individual is 0).

Diversity values for the range of initial communities were from 3.7324 for the least even community (var = 100) to 6.9078 for the perfectly even community (Fig. 4). Note that for the even community, dilution caused no change in H' until the number of individuals in the community reached the number of species in the community, in this case 1×10^3 individuals, corresponding to 1×10^3 types at the 10^{-3} dilution. Upon dilution, little change in H' was observed when the number of individuals greatly exceeded the number of species; however, H' decreased to the theoretical value of 2.3026 (based on H'_{\max} for the even distribution) at a dilution of 10^{-5} . The sole exception was in the community

Evenness of the communities not initially perfectly even increased with dilution and reached the theoretical maximum of 1.0 at the 10^{-5} dilution (Fig. 5). In the community obtained by setting $\text{var} = 20,000$, there was a change in evenness that was not consistent with the patterns displayed by dilution of the other communities. Because the communities were formed by probabilistic sampling, it is possible that the anomaly is simply a variant, although it is not understood why the evenness obtained did not seem to influence subsequent dilutions.

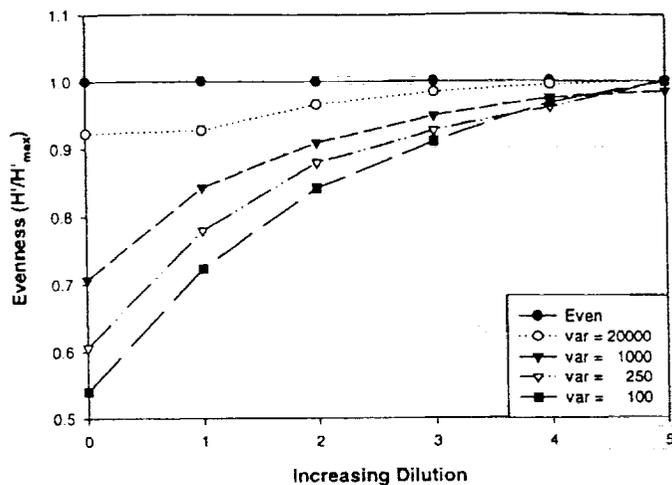


Fig. 5. Changes in evenness with dilution in communities of differing initial evenness.

species lost in the first dilution decreased substantially and approached the theoretical value of no species lost in the first dilution. Furthermore, in the treatment $\text{var} = 20,000$, loss of species in subsequent dilutions was also low, comparing favorably with the perfectly even state.

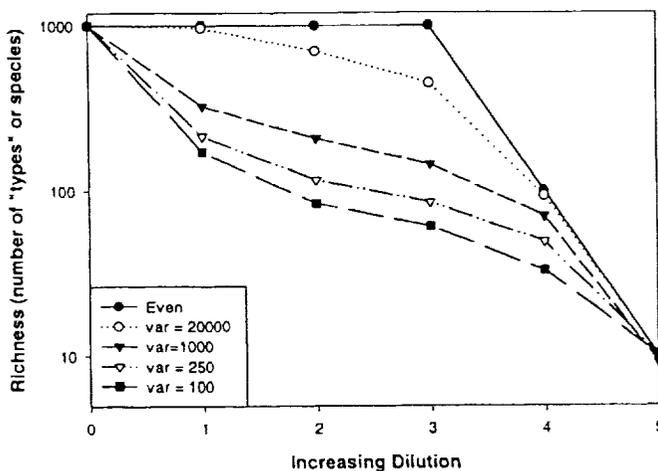


Fig. 6. Changes in richness with dilution in communities of differing initial evenness

Richness of communities changed differently with dilution depending on the initial evenness (Fig. 6). For communities of low initial evenness, richness dropped rapidly with the first dilution. As the initial community became more even, the number of

species lost in the first dilution decreased substantially and approached the theoretical value of no species lost in the first dilution. Furthermore, in the treatment $\text{var} = 20,000$, loss of species in subsequent dilutions was also low, comparing favorably with the perfectly even state.

Incubation Experiments

Raw sewage was collected from the Cape Canaveral Air Station Waste Water Treatment Facility (Kennedy Space Center, FL). Samples were allowed to settle for ~2 hours (to remove large particles) and a serial dilution (through 10-6) was prepared from the supernatant to use as the inoculum for the batch culture experiments. Probability suggests that, because the dilution of the original community should remove rare organisms, these inocula should systematically differ in diversity (both richness and evenness).

Seven treatments (undiluted (100) through 10-6) were established by adding 1 ml of inoculum to 60 ml of sterile sewage in a 125-ml flask. For each treatment, three replica flasks were maintained; all flasks were kept on a shaker table (150 rpm) to ensure aerobic conditions. Each day, 20 ml of liquid were removed from each flask and replaced with 20 ml of sterile sewage.

After 9 days (3 retention times), flasks were harvested and samples collected for total cell counts (Acridine Orange direct counts), culturable counts on R2A agar, richness, evenness, and

diversity of colony morphologies on R2A, community level physiological profiling (CLPP (5)), dilution-extinction analysis of CLPP (4), molecular analysis of whole community DNA, (including AFLP (16) and T-RFLP (8))

Richness, evenness, and diversity of colony morphologies on R2A

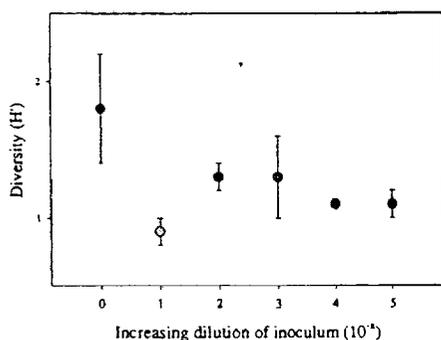


Fig. 7. A decrease in diversity (based on colony morphology on R2A agar) was observed with increasing dilution. The X-axis shows the exponent of the dilution factor used in the original inoculum (e.g. "4" is the regrown community derived from the 10^{-4} dilution inoculum). The Y-axis is diversity (Shannon-Wiener index), and error bars shown are \pm one standard deviation.

For each treatment, a serial dilution of the regrown community was plated onto R2A agar and incubated at room temperature. After 72 hours, 25 colonies were randomly chosen from each countable plate and colony morphology described. Richness (S) was then determined as the total number of colony types encountered (Fig. 8); evenness (E) (Fig. 8) and diversity (H') (Fig. 7) were calculated using the Shannon-Wiener diversity index.

T-RFLP analysis of bacterial 16S rRNA genes

Terminal restriction fragment length polymorphism (T-RFLP) was used to compare the overall diversity of the bacterial communities from these flasks. The bacterial 16S rRNA gene was amplified using two primers (1392 Reverse (5'ACGGGCGGTG TGTRC) and 8 Forward (5'AGAGTTTGATCCTGGCTCAG (labeled)));

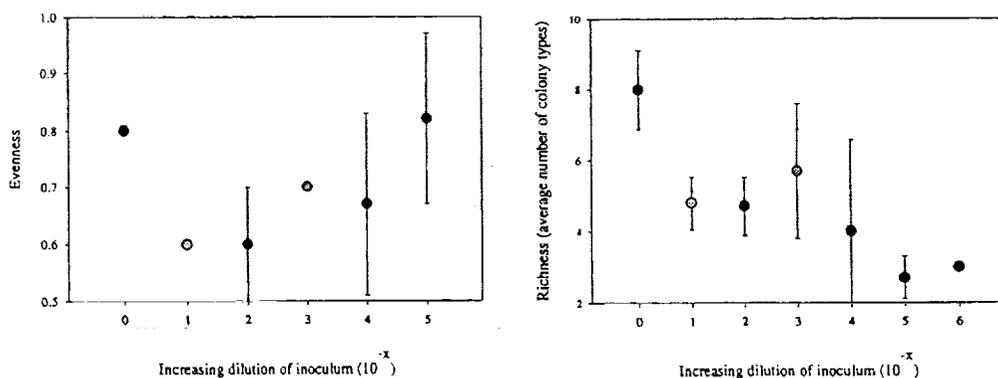


Fig. 8. An increase in overall community evenness and a decrease in richness (number of colony types) was observed with increasing dilution. The X-axis shows the exponent of the dilution factor used in the original inoculum, and error bars shown are \pm one standard deviation.

aliquots of the PCR product were then digested using either the HhaI or MspI restriction enzyme. Using an automated DNA sequencer, the length of the terminal restriction fragments was then compared across the different communities. HhaI generated 43 different T-RFLP fragments (the average sample contained 16 fragments) and MspI generated 42 different fragments (the average sample contained 12). The number of fragments observed across the dilution/diversity gradient did not differ (Table 1), though one might have expected a decrease corresponding to a loss in

diversity (richness). Additionally, principle components analysis (PCA) was performed on the combined (MspI and HhaI) datasets (Fig. 9).

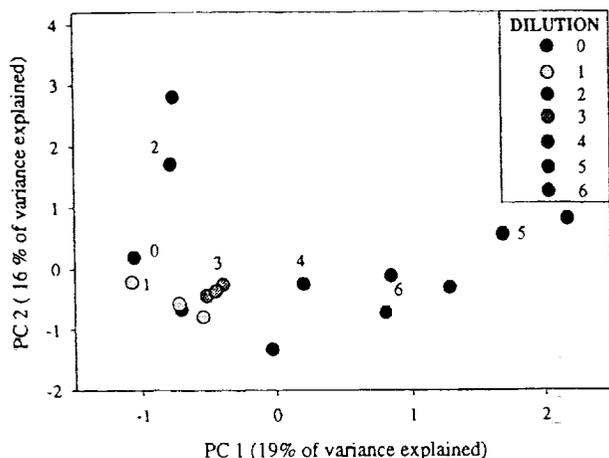


Fig. 9. PCA diagram of the T-RFLP fingerprints. Each "dilution" identified in the legend is the exponent of the dilution used in the original inoculum (e.g. "4" represents the regrown community inoculated with the 10^{-4} dilution). On the graph, the high dilution (theoretically low diversity) treatments 5 and 6 separated from all the other treatments on the first principle component.

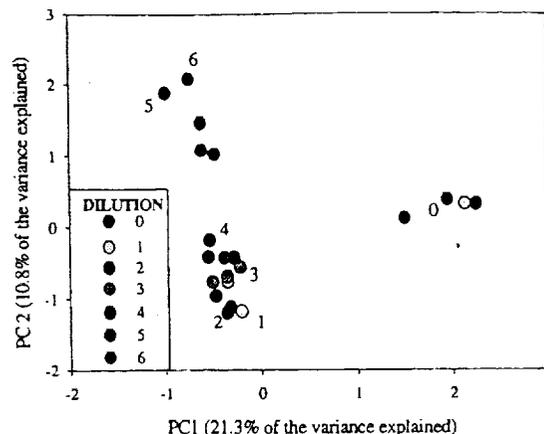


Fig. 10. PCA of the AFLP fingerprints. Each "dilution" identified in the legend is the exponent of the dilution used in the original inoculum (e.g. "4" represents the regrown community inoculated with the 10^{-4} dilution). This graph shows that the different dilution/diversity inocula separated into three main groups based on overall genetic structure. The undilute inocula ("0") were most distinct, separated on PC1, and the very dilute inocula ("5" and "6") were distinct from all others on PC2. Cluster analysis was also performed on the AFLP data and the same major divisions were observed (data not presented).

Fingerprinting of whole-community DNA using AFLP

AFLP (Amplified Fragment Length

Polymorphism) was used to profile overall community structure in this experiment. AFLP is based on amplification of DNA restriction fragments, after ligation of an adapter sequence, using fluorescently labeled PCR primers (4). It is similar to other "arbitrarily primed" PCR based fingerprinting techniques because it requires no *a priori* sequence information for primer design (unlike T-RFLP); it differs in that it is more reproducible and provides a greater resolution with fewer primers. In this research, three different primer sets were used in the amplification, and the data pooled (85 bands). The presence or absence of each band in each sample was recorded and analyzed using principal components (Fig. 10) and cluster analyses (data not shown).

The average number of AFLP bands per treatment decreased along the dilution/diversity gradient (Table 1). Because AFLP fragments are, in principle, simply RFLPs resolved by selective PCR amplification of the DNA restriction fragments, the number of fragments produced can be expected to correlate with the overall diversity of the communities. Furthermore, most of the bands encountered in the undilute ("0") treatment were unique (not found in any further treatments), corresponding to a large change in community structure (perhaps richness) after the first dilution.

Community level physiological profiling

Community level physiological profiling (CLPP) was used to compare overall functional potential among the different dilution/diversity treatments based on each community's ability to metabolize 95 different sole carbon sources (1). Principle components analysis was used to

analyze the data and the first two PCs were plotted as a means of visualizing the relationships among the different samples (Fig 11).

Table 1. Summary of diversity analysis for plates and molecular techniques

Dilution	R2A plates		AFLP		T-RFLP			
	Avg. # of colony types	Proportion unique ¹	Avg. # of bands	Proportion unique ²	# bands w/ MspI	Proportion unique ³	# bands w/ HhaI	Proportion unique
10 ⁰	8	0.4	26	0.9	7	0.21	19	0.07
10 ⁻¹	4.8		24		9		15	
10 ⁻²	4.7	0	17	0.6	11	0.21	20	0.23
10 ⁻³	5.7		17		10		12	
10 ⁻⁴	4		14		11		13	
10 ⁻⁵	2.7		20		18		21	
10 ⁻⁶	3	1.0	15	0.5	8	0.17	10	0.19

1 - Number of unique colony types in a treatment group/total number of colony types encountered in each treatment group. "Unique" refers to a band (colony type, etc.) that is present a particular treatment group (e.g. 10⁰ and 10⁻¹) that is not present in either of the other two treatment groups. It is interesting to note that all of the colony morphologies observed in the 10⁻⁶ treatment were unique (not encountered in any of the higher dilution/diversity treatments). Nearly half (40%) of the colony morphologies encountered in the low dilution/high diversity treatment were unique while none of the colony types encountered in the mid-dilutions were unique (all of those colony types were encountered in these treatments were also observed in the 10⁰ and 10⁻¹).

2 - Number of unique AFLP bands in a treatment group/total number of AFLP bands encountered in each treatment group. Nearly all of the bands encountered in the low dilution/high diversity treatments were unique suggesting many organisms types were present in this treatment and diluted out of others.

3 - Number of unique T-RFLP bands in a treatment group/total number of T-RFLP bands encountered in each treatment group. The majority of the T-RFLP peaks were not unique, and were found across the entire dilution series. This suggests a large bias in the T-RFLP/PCR procedure towards certain organisms. If we assume these organisms to be dominant, then these results suggest that the same types of organisms were dominate throughout the dilution series. It is also interesting to note that number of T-RFLP peaks (number of ribotypes) was significantly greater than the number of colony morphologies on R2A agar.

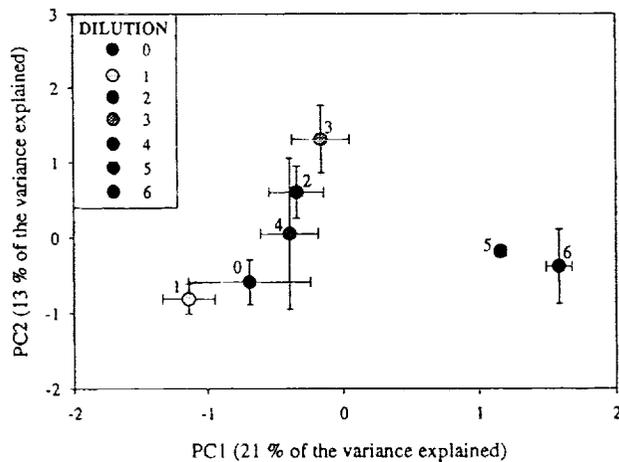


Fig. 11. PCA of the CLPP. Each "dilution" identified in the legend is the exponent of the dilution used in the original inoculum (e.g. "4" represents the regrown community inoculated with the 10⁻⁴ dilution). Error bars represent 95% confidence intervals around the centroid for each treatment (average of 3 independent replica flasks). Two distinct groups can be distinguished on this plot; treatments 0 through 4 (undiluted through 10⁻⁴) form one group separate from the 10⁻⁵ and 10⁻⁶ dilution communities.

Dilution-extinction analysis of CLPP profiles

Garland and Lehman (4) used the extinction of functional characters in CLPP assays to make inferences about diversity of microbial communities. Plotting the number of positive tests obtained in CLPP analyses of dilutions made of each regrown community against the number of cells inoculated into each well of the BIOLOG plate yields curves that resemble those obtained in saturation plots. Fitting a rectangular hyperbola to the data produces two parameters that are analogous to those obtained with saturation plots:

$$F = \frac{F_{\max} \times K_F}{1 + K_F} \quad (3)$$

where F is the number of positive tests at a given inoculum density (referred to by Garland and Lehman as the functional richness), F_{\max} is the estimated maximum number of tests that would be positive for the community, and K_F is the cell density at which $F = F_{\max}/2$. Parameter estimates and the curves representing each dilution treatment are shown in Fig. 12.).

Differences in microbial community structure were detected throughout the dilution/diversity gradient with every measure employed. Major groups formed as follows: the undilute, regrown communities ("0" (high diversity?)), the very dilute (10^{-5} and 10^{-6} (very low diversity?)), and all others (10^{-1} through 10^{-4}).

Conclusions

- Different measures (which focused on different elements of "diversity" and had different inherent methodological biases) showed different levels of resolution. For example:

- comparing culturable diversity (richness and evenness) of colony types of R2A highlighted the difference between the undilute inocula and all others
- PCA analysis of CLPP showed that the 10^{-5} and 10^{-6} treatments were different from all others, but did not separate the undilute community. Dilution/extinction analysis of CLPP divided the communities into the three dilution/diversity groups.
- PCA of the T-RFLP profiles showed that the 10^{-5} and 10^{-6} treatments were different from all others, though no change in diversity (measured by the relative number of peaks) was observed.
- AFLP divided the communities into three dilution/diversity groups (using PCA) based on overall structure.

Interpreting all of these results in the context of the numerical simulations, it seems that the original community was highly dominant (e.g. var = 100 or var = 250). For these curves, the greatest difference in diversity was observed after the initial dilution (loss of richness) and at extreme dilutions (10^{-5}); these theoretical divisions agree well with the experimental results. Assuming a highly dominant initial community, measures that showed the undilute regrown community to be unique (R2A colony morphology, AFLP and dilution/extinction of CLPP) probably were better at detecting differences in richness. Measures that showed the 10^{-5} and 10^{-6} treatments to be unique (T-RFLP, AFLP, PCA of CLPP, dilution/extinction of CLPP) probably reflected differences in overall diversity (richness and evenness) better. It is interesting to note

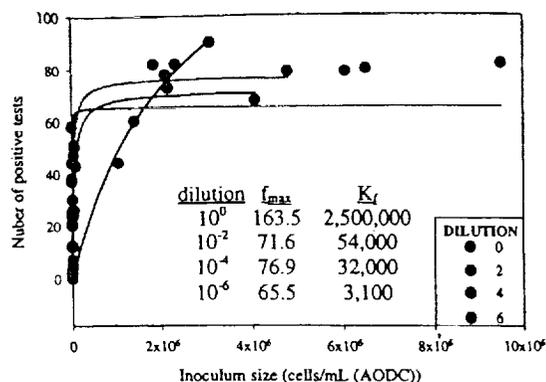


Fig. 12. Treatments 0, 2, 4, and 6 were profiled using dilution/extinction analysis. The raw data are presented (number of positive characters versus cell concentration) and fit with a rectangular hyperbola; parameter estimates for this fit are given in the legend. The general shapes of each dilution/extinction curve were used by Garland and Lehman to suggest differences in communities based on the dominance of generalists vs specialists. In this figure, the curve for the undiluted inoculum ("0") suggests a community dominated by specialists, where rapid loss of strains also means a rapid loss of characters. In the higher dilution treatments, the lower values of F_{\max} and the lower values of K_f imply a community composed of generalists in which there are fewer functions, but in which those functions are conserved throughout the community. An alternate explanation is that more even communities tend to lose strains (and by implication, functions) more slowly through dilution.

that nearly all measures (except R2A) showed the 10^{-5} and 10^{-6} treatments to be unique, not all measures separated the undiluted regrown community.

Although the use of dilution to produce communities of different (lower) diversity is a reasonable means of manipulating diversity, the effects are non-linear. When dilution is coupled with the additional non-linear effects of regrowth of mixed cultures, a very complex result is obtained. Clearly much further work is needed to determine the extent to which the effects can be predicted and controlled for experimental and applications purposes.

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